Tissue-specific expression and subcellular localisation of mammalian δ-tubulin

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The properties of the microtubule network are regulated at various levels including tissue-dependent isotype switching, post-translational modification of α- and β-tubulin, and by a variety of microtubule-associated molecules (for reviews, see [1–3]). Microtubule nucleation is attributed to γ-tubulin, which is present in protein complexes at the centrosome and in the cytoplasm [4,5]. A screen for flagellar mutants in the green alga *Chlamydomonas reinhardtii* has led to the identification of a fourth member of the tubulin gene superfamily, δ-tubulin. In this unicellular organism, the lack of a functional δ-tubulin gene copy causes aberrant numbers of flagella, depending on the age of the corresponding basal bodies; mutants also show abnormal ultrastructure of the basal bodies and a misplacement of the cleavage furrow at mitosis [6]. Here, we report the isolation of the mouse δ-tubulin homologue and show that the gene is highly expressed in testis. In the elongating spermatid, δ-tubulin associated with the manchette, a specialised microtubule system present during reshaping of the sperm head. The protein specifically localised at the perinuclear ring of the manchette, at the centriolar vaults and along the principal piece of the sperm flagellum. In somatic cell lines, unlike most other tubulins, mammalian δ-tubulin was both cytoplasmic and nuclear and did not colocalise with microtubules. The protein was enriched at the spindle poles during mitosis and we found that γ-tubulin coimmunoprecipitated with δ-tubulin. Together, the data indicate a specialised role for mammalian δ-tubulin that is distinct from other known tubulins.

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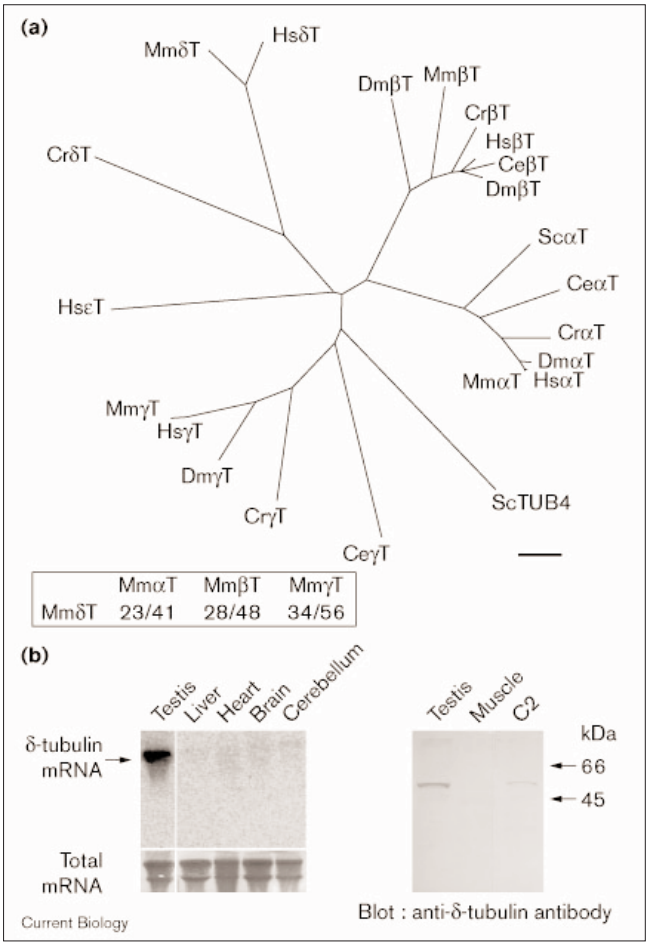
Results and discussion

The mouse δ-tubulin cDNA was isolated by reverse transcription (RT)–PCR from an embryonic day 13.5 (E13.5) cDNA library (see Materials and methods). The deduced peptide sequence showed 43% and 36% identity with the amino- and carboxy-terminal regions, respectively, of the *C. reinhardtii* δ-tubulin sequence. The mouse and the recently reported human δ-tubulin cDNA [7] lack the sequence corresponding to exons 6 and 7 (which code for amino acids 233–388) of the *Chlamydomonas* gene, a region with no significant homology to other known tubulin genes. Figure 1a displays the phylogenetic relationship between mammalian δ-tubulin and other tubulin genes. Remarkably, no δ-tubulin sequences have been identified in the genomes of the yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, the centrosome equivalents of which have no centrioles. The gene has not been found in the genome of the nematode *Caenorhabditis elegans*, which has aflagellate spermatids, either. High levels of δ-tubulin mRNA were detected in mouse testis by northern blot analysis (Figure 1b, left panel) whereas, in differentiated tissues such as liver, heart or brain, δ-tubulin mRNA was detectable only at low levels. Together, these findings suggest a specialised role of the mammalian δ-tubulin gene either in spermiogenesis or in centrioles of somatic cells.

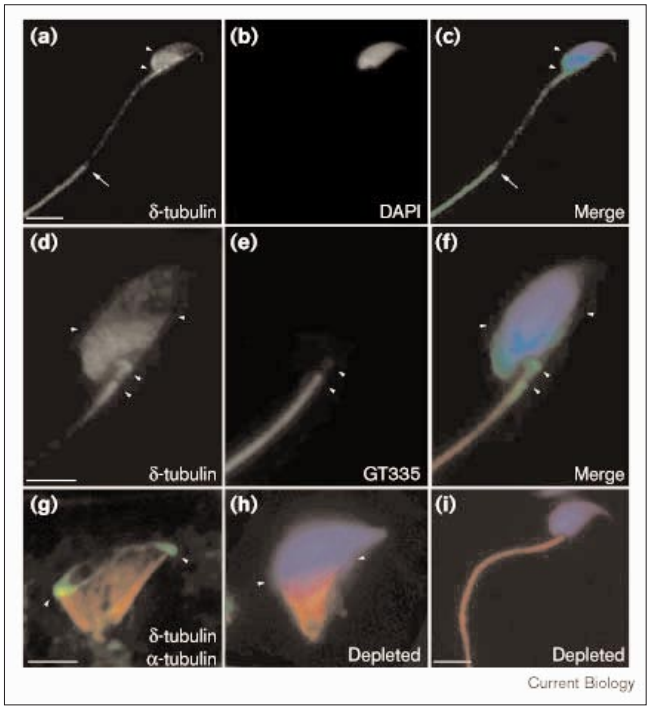
Given the flagellar phenotype and the ultrastructural changes of basal bodies in *Chlamydomonas uni3–1* mutants [6], and the high expression levels in mouse testis (Figure 1b), we considered that either spermatids or, more generally, tissues with a high proportion of dividing cells represented a good candidate cell type in which the role of mammalian δ-tubulin could be investigated. A polyclonal antibody was raised against recombinant mouse δ-tubulin, affinity purified and tested on western blots where a band of the predicted size of 51 kDa was detected in mouse testis and exponentially growing C2 myoblasts (Figure 1b, right panel). Figure 2 shows the immunofluorescence patterns that were obtained on epididymal sperm and testicular spermatids. In the majority of epididymal sperm, the antibody stained the caudal part of the sperm head, the connecting piece (Figure 2d,f; arrowhead) and the principal piece of the flagellum (Figure 2a,c; arrow). The signals resisted detergent extraction and covered the area of the sperm head which, in earlier stages of spermiogenesis, is occupied by the manchette. This cytoskeletal structure represents a transient microtubule array unique to spermatids that might participate in reshaping of the sperm nucleus [8,9]. In testis-derived elongating spermatids, in which the microtubule manchette was still present, the anti-δ-tubulin antibody specifically marked the perinuclear ring (Figure 2g). This ring structure has previously been described at the ultrastructural level [8] and, recently,

Figure 1



(a) Phylogenetic tree showing the relative protein sequence similarity between various representatives of the tubulin gene superfamily. The branches show the clustering of the δ-tubulin genes. Tubulins from a selected panel of model organisms are included. Cr, *C. reinhardtii*; Hs, human; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*. Ce, *Caenorhabditis elegans*. The table gives the percentage identity/similarity for mouse δ-tubulin when compared with mouse α-, β- and γ-tubulin. The bar represents 0.1 substitutions per site. **(b)** Left panel, a mouse δ-tubulin cDNA probe detected a strong signal at 1.7 kb on a northern blot containing total RNA from various organs. Right panel, an affinity-purified polyclonal antibody raised against recombinant mouse δ-tubulin detected a band at 51 kDa in protein extracts from testis and C2 myoblasts, whereas no signal was detected in muscle (each lane contains 15 μg total protein).

Figure 2



Immunolocalisation of δ-tubulin on epididymal sperm and testicular mouse spermatids. **(a–f)** The purified antibody marked the caudal part of epididymal sperm heads (arrowheads, upper pair in panels a,c,d,f; green in panels c,f), the centriolar vaults (arrowheads, lower pair in panels d,f; green in panel f) and at the principal piece of the flagellum (arrow in panels a,c; green in panel c). In contrast, antibodies directed against (e,f) polyglutamylated tubulin (GT335; red in panel f) or α-tubulin (red in panel i) stained the middle piece of the flagellum continuously. **(g)** In testis-derived sperm manchettes, anti δ-tubulin antibody (green) marked the perinuclear ring. **(h,i)** The green signal obtained with the anti δ-tubulin antibody was suppressed when the antibody was pre-adsorbed with recombinant δ-tubulin (depleted); (h) testicular spermatids, (i) epididymal sperm. In (g–i), α-tubulin staining is red. DAPI, 4,6-diamidino-2-phenylindole. The scale bars represent 5 μm.

biochemical fractionation has suggested the presence of tubulins, β-actin and a yet unknown protein copurifying with this structure [10]. In the developing spermatid, δ-tubulin constitutes a component of the perinuclear ring localised at the apical aperture of the manchette.

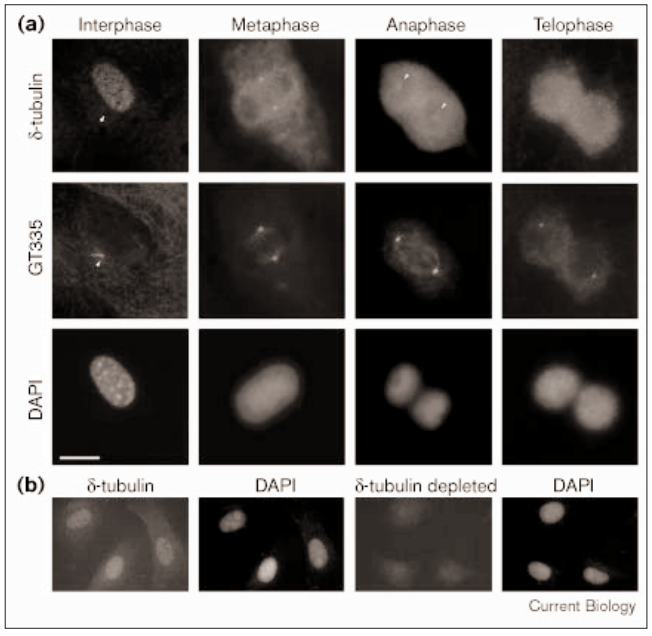
At the end of mouse spermiogenesis, first the distal and later the proximal centriole degenerate and leave behind a centriolar vault in mature sperm [11,12]. The anti-δ-tubulin antibody recognised the proximal and distal area of the centriolar vault from which the signal faded into the axoneme (Figure 2a–f). Remarkably, this staining pattern was also found in mature sperm where centrioles have degenerated. Thus, δ-tubulin is probably not an integral part of the centrioles *per se* but, rather, is centriole-associated. As for the absence of a signal along the middle piece, the possibility of restricted epitope accessibility in this segment of the flagellum cannot be ruled out. Under our permeabilisation and fixation conditions, however, coimmunostaining using monoclonal antibodies directed either against α-tubulin or polyglutamylated tubulin [13] marked the axoneme continuously throughout the middle piece (Figure 2e,f,i). A clear anti-δ-tubulin signal was observed along the principal piece of the flagellum (Figure 2a,c; arrow). In this segment, the axoneme is surrounded by a filamentous structure known as the fibrous sheath [14]. Further analysis at the ultrastructural level should confirm

whether δ-tubulin is a component of the fibrous sheath rather than of the axoneme.

The perturbed basal body structure in *C. reinhardtii uni3–1* mutants and the conservation of the centriole ultrastructure in mammals suggest a common role for δ-tubulin in these structures. To determine whether δ-tubulin protein is directly associated with centrioles in somatic cells, its distribution was studied in exponentially growing C2 myoblasts expressing δ-tubulin (Figure 1b, right panel) and using the anti-δ-tubulin antibody in combination with a monoclonal antibody directed against polyglutamylated tubulin as a centriole marker (Figure 3). The general cytoplasmic staining did not show any colocalisation with the microtubule network. In addition to the cytoplasmic signal, a diffuse nuclear signal was observed and became more pronounced after detergent extraction of the cytoplasm (compare Figure 3a with Figure 3b). The presence of tubulin in the nuclear compartment of somatic cells is unexpected but has also been reported for the βII isotype of tubulin [15]. It remains to be determined how δ-tubulin, which does not contain any obvious nuclear localisation sequence, associates or enters into the nucleus.

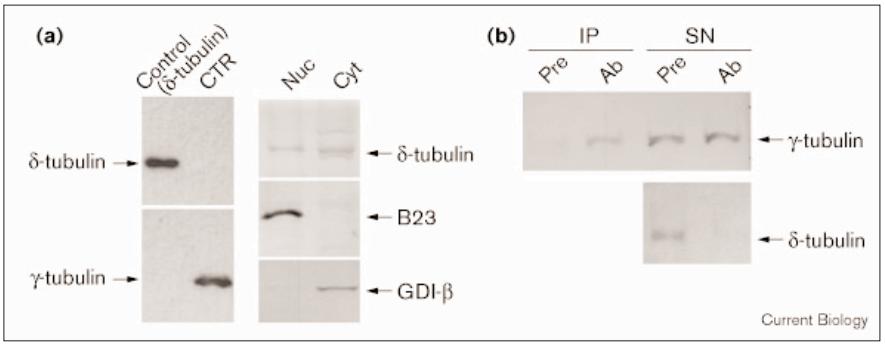
C2 myoblasts showed neither δ-tubulin staining at the centrioles nor in the centrosome area during interphase (Figure 3a, first column). This result is at variance with a recent report on the human osteosarcoma cell line U2OS, which suggested an intercentriolar accumulation of human δ-tubulin [7]. An immunodepletion control using recombinant δ-tubulin competed with the signal seen in interphase and mitotic cells (Figure 3b). Furthermore, we confirmed by western blot analysis that centrosomes isolated from a human lymphoblastoid cell line did not contain any detectable amount of δ-tubulin (Figure 4a, left panel). This experiment also ruled out a possible cross reactivity of the anti-δ-tubulin antibody with other tubulins, which are abundant in centrosome preparations [16].

Figure 3



(a) Localisation of δ-tubulin in C2 myoblasts during various cell-cycle stages. Cells were stained using anti-δ-tubulin antibody (first row) and the centriole marker GT335 (second row), and nuclei stained using DAPI (third row). After detergent extraction, a signal was seen at the nucleus but could not be detected at the centrosomes during interphase in this cell type (the position of the centrioles is marked by arrowheads in the first and third column). At metaphase and anaphase, a signal was detected at the spindle poles, but disappeared at telophase/cytokinesis. **(b)** Pre-adsorption of the antibody with recombinant δ-tubulin reduced the staining in interphase nuclei. Panel 1, pre-adsorption against BSA; panel 3, pre-adsorption against recombinant δ-tubulin; panels 2 and 4, DAPI. The scale bar represents 5 μm.

Figure 4



(a) Presence of γ-tubulin (lower left panel) and absence of δ-tubulin (upper left panel) in the centrosome fraction from lymphoblastoid cells (CTR). The control lane was loaded with recombinant δ-tubulin. In myoblasts, δ-tubulin could be detected in the nuclear (nuc) and in the cytoplasmic (cyt) fractions. The antibodies used are indicated on the side of each blot and the arrows indicate the corresponding migration positions of the proteins. **(b)** Coimmunoprecipitation of δ-tubulin with a minor fraction of γ-tubulin in C2 myoblasts. Immunoprecipitation was performed using either purified anti δ-tubulin (Ab) or a preimmune control (Pre). IP, immunoprecipitate; SN, supernatant. Blots were stained using a monoclonal anti-γ-tubulin antibody (upper panel) or a polyclonal anti-δ-tubulin antibody (lower panel).

A weak centrosome signal appeared only at the spindle poles during mitosis (Figure 3). In agreement with the immunolocalisation data, western blot analysis showed the presence of δ-tubulin in both cytoplasmic and nuclear compartments (Figure 4a, right panel). The differences between the results obtained in the osteosarcoma-derived cell line [7] and interphase myoblasts (this work) could be explained in several ways. One possibility would be that the presence of δ-tubulin at the centrosome depends on tissue, differentiation stage or cell-cycle stage. Alternatively, the detectability of the centrosome could depend on distinct epitope accessibilities for the respective antibodies used. A screen of various tissue and cell types for centrosomal signals together with ultrastructural δ-tubulin localisation studies will allow investigation of the functional role of this protein in specialised cell types. Whether the protein could play a role in regulating microtubule dynamics was addressed in a coimmunoprecipitation experiment where a minor fraction of γ-tubulin was found to be associated with δ-tubulin (Figure 4b). This finding suggests a role for δ-tubulin in regulating γ-tubulin-associated functions such as microtubule nucleation [5] or centriole/basal body duplication [17].

Whereas the *C. reinhardtii UNI3* gene, which encodes δ-tubulin, appears to be involved in flagellogenesis, basal body structure and cell division, we propose that, in mammals, one major function of this gene might be tissue-specific. As shown in the present work, mouse δ-tubulin specifically associates with the perinuclear ring of the microtubule manchette, a unique cytoskeletal structure which is found in the developing spermatid. Both the manchette and the mitotic spindle apparatus are transient structures where δ-tubulin could play a role. In the soluble cytoplasmic pool, δ-tubulin associates with γ-tubulin and might be involved in regulating γ-tubulin function by direct or indirect interaction with this molecule. The search for other proteins interacting with δ-tubulin and the identification of further δ-tubulin-expressing tissues or differentiation stages will help us understand the general role of this new tubulin, which is conserved between green algae and mammals.

Supplementary material

Supplementary material including supplementary methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

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